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(54) Title: NOVEL VACCINE COMPOSITION

(57) Abstract: The invention provides a vaccine composition comprising the toxoplasma protein, SAG3 or an immunogenic derivative thereof in combination with a suitable adjuvant and/or carrier. The invention further provides a vaccine composition which additionally comprises the toxoplasma protein, SAG1 or an immunogenic derivative thereof, or a truncated SAG1 comprising amino acid residues 48-307 of SAG1.

NOVEL VACCINE COMPOSITION

DESCRIPTION

Toxoplasma gondii is an obligate intracellular protozoan parasite responsible for toxoplasmosis in warm-blooded animals, including man. Although it is generally clinically asymptomatic in healthy individuals, toxoplasmosis may cause severe complications in pregnant women and immunocompromised patients (1-4).

A live attenuated S48 Toxoplasma strain of the parasite is currently available for vaccination in sheep (Toxovax, Mycofarm) (5). However, this vaccine cannot be administered to humans because of possible reversion to virulent forms. The development of subunit vaccines thus constitutes an alternative way to achieve effective protection of humans against congenital infection and to prevent infection of immunosuppressed individuals.

SAG3 is a 43 kDa surface protein expressed in all invasive stages (6). Like SAG1 (see PCT application number PCT/ EP99/ 03957), SAG3 is anchored to the membrane via a glycosylphosphatidylinositol anchor (7). Members of the SAG1 family of proteins are thought to be involved in interactions with the host cell, as antibodies against SAG1 block invasion. Several lines of evidence indicate that Toxoplasma tachyzoites interact with host cells by specific receptor-ligand types of interactions. For instance, invasion of host cells by tachyzoites can be inhibited by treating the parasite with rabbit polyclonal and mouse antibodies against the major surface protein SAG1 (8-10). However, the fact that a SAG1-deficient mutant is still able to infect host cells suggests that additional parasite molecules may be available to allow host cell infection (9).

The gene encoding SAG3 has been cloned and sequenced (6). A homology of 24% was found between the predicted amino acid sequence of SAG3 and that of SAG1. The conservative distribution of the cysteine and tryptophan residues, their similar hydrophobic-philic profiles as well as of repeated motifs (Q-Y-C-S-G and G-A-T-L-T-I) suggest similar folding for both proteins. Mutants lacking the SAG3 protein are 1,000-fold less virulent and invade host cells with 50% of the efficiency of their wild-type counterparts (11).

The sequence encoding the SAG3 protein (385 amino acid residues) was recovered by PCR amplification (as described in Example 1). This sequence (SEQ ID No. 1) shows some differences from the sequence found previously by Cesbron et al (6) (see Figure 1). 17 nucleotide bases differed from the sequence of Cesbron et al and the amino acid sequences showed 97.4% homology, differing by 10 amino acids.

The present invention provides a vaccine composition comprising the toxoplasma protein, SAG3, or an immunogenic derivative thereof, in combination with a suitable adjuvant and/or carrier.

The invention further provides a SAG3 protein comprising the amino acid sequence of SEQ ID No: 2, or an immunogenic derivative thereof. The invention preferably provides a truncated SAG3 protein, preferably purified, especially one in which the anchor region of SAG3 is absent.

In one aspect of the invention the truncated, anchor-less SAG3 protein is lacking amino acids 360 – 385 of the SAG3 protein.

In a further aspect of the invention there is provided a truncated SAG3 protein comprising amino acids 40 – 359 of SAG3, and immunogenic derivatives thereof.

The term "immunogenic derivative" encompasses any molecule such as a truncated or other derivative of the protein which retains the ability to induce an immune response to the protein following internal administration to a human or to an animal or which retains the ability to react with antibodies present in the sera or other biological samples of Toxoplasma *gondii*-infected humans or animals. Such immunogenic derivatives can be prepared by the addition, deletion, substitution or rearrangement of amino acids or by chemical modifications thereof.

The present invention further provides a vaccine composition comprising the toxoplasma protein of SEQ ID No:1 or an immunogenic derivative thereof, in combination with a suitable adjuvant and/or carrier.

More preferably, the vaccine composition of the invention comprises a truncated SAG3 protein comprising amino acids 40 – 359 of SAG3, or an immunogenic derivative thereof, in combination with a suitable adjuvant and/or carrier.

In a further aspect, the present invention provides a vaccine formulation as herein described for use in medical therapy, particularly for use in the treatment or prophylaxis of toxoplasmosis infections. The vaccine formulation will be useful in the prevention of both horizontal and vertical (congenital) transmission of toxoplasmosis.

The vaccine compositions according to the invention will normally comprise a protein according to the invention, as described hereinabove, admixed with a suitable adjuvant and/or carrier.

The vaccine composition according to the invention may comprise further components for the treatment or prophylaxis of infections other than toxoplasmosis infections. In particular such further components may be one or more antigens from one or more other pathogens.

In a further aspect there is provided a vaccine composition according to the invention comprising one or more additional *T. gondii* antigens, for example TG34 as described in WO 92/11366 or SAG1 as described in PCT/EP99/03957. A preferred composition includes a SAG3 protein as described together with a SAG1 protein or immunogenic derivative thereof. A preferred SAG1 protein for use in the invention is a truncated SAG1 protein in which the anchor region of SAG1 is absent. The truncated SAG1 protein most preferably comprises amino acids 48 –307 of SAG1, and immunogenic derivatives thereof. Such SAG1 proteins are preferably expressed in a *Pichia Pastoris* expression system.

The vaccine of the present invention will contain an immunoprotective or immunotherapeutic quantity of the antigen and may be prepared by conventional techniques.

Vaccine preparation is generally described in New Trends and Developments in Vaccines, edited by Voller et al., University Park Press, Baltimore, Maryland, U.S.A. 1978. Encapsulation within liposomes is described, for example, by Fullerton, U.S. Patent 4,235,877. Conjugation of proteins to macromolecules is disclosed, for example, by Likhite, U.S. Patent 4,372,945 and by Armor et al., U.S. Patent 4,474,757.

The amount of protein in the vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccines. Such amount will vary depending upon which specific immunogen is employed. Generally, it is expected that each dose will comprise 1-1000 μg of protein, preferably 2-100 μg, most preferably 4-40 μg. An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of antibody titres and other responses in subjects. Following an initial vaccination, subjects may receive a boost in about 4 weeks.

The proteins of the present invention are preferably adjuvanted in the vaccine formulation of the invention. Adjuvants are described in general in Vaccine Design – the Subunit and Adjuvant Approach, edited by Powell and Newman, Plenum Press, New York, 1995.

Suitable adjuvants include an aluminium salt such as aluminium hydroxide gel (alum) or aluminium phosphate, but may also be a salt of calcium, iron or zinc, or may be an insoluble suspension of acylated tyrosine, or acylated sugars, cationically or anionically derivatised polysaccharides, or polyphosphazenes.

In the formulation of the invention it is preferred that the adjuvant composition induces a preferential Th1 response. However it will be understood that other responses, including other humoral responses, are not excluded.

Preferred Th1-type immunostimulants which may be formulated to form adjuvants suitable for use in the present invention include and are not restricted to the following.

Monophosphoryl lipid A, in particular 3-de-O-acylated monophosphoryl lipid A (3D-MPL), is a preferred Th1-type immunostimulant for use in the invention. 3D-MPL is a well known adjuvant manufactured by Ribi Immunochem, Montana. Chemically it is often supplied as a mixture of 3-de-O-acylated monophosphoryl lipid A with either 4, 5, or 6 acylated chains. It can be prepared by the methods taught in GB 2122204 B. A preferred form of 3D-MPL is in the form of a particulate formulation having a small particle size less than 0.2μm in diameter, and its method of manufacture is disclosed in EP 0 689 454.

Saponins are also preferred Th1 immunostimulants in accordance with the invention. Saponins are well known adjuvants and are taught in: Lacaille-Dubois, M and Wagner H. (1996. A review of the biological and pharmacological activities of saponins. Phytomedicine vol 2 pp 363-386). For example, Quil A (derived from the bark of the South American tree Quillaja Saponaria Molina), and fractions thereof, are described in US 5,057,540 and "Saponins as vaccine adjuvants", Kensil, C. R., *Crit Rev Ther Drug Carrier Syst*, 1996, 12 (1-2):1-55; and EP 0 362 279 B1. The haemolytic saponins QS21 and QS17 (HPLC purified fractions of Quil A) have been described as potent systemic adjuvants, and the method of their production is disclosed in US Patent No. 5,057,540 and EP 0 362 279 B1. Also described in these references is the use of QS7 (a non-haemolytic fraction of Quil-A) which acts as a potent adjuvant for systemic vaccines. Use of QS21 is further described in Kensil *et al.* (1991. J. Immunology vol 146, 431-437). Combinations of QS21 and polysorbate or cyclodextrin are also known (WO 99/10008). Particulate adjuvant systems comprising fractions of QuilA, such as QS21 and QS7 are described in WO 96/33739 and WO 96/11711.

Another preferred immunostimulant is an immunostimulatory oligonucleotide containing unmethylated CpG dinucleotides ("CpG"). CpG is an abbreviation for cytosine-guanosine dinucleotide motifs present in DNA. CpG is known in the art as being an adjuvant when administered by both systemic and mucosal routes (WO 96/02555, EP 468520, Davis *et al.*, *J.Immunol*, 1998, 160(2):870-876; McCluskie and Davis, *J.Immunol.*, 1998, 161(9):4463-6). Historically, it was observed that the DNA

fraction of BCG could exert an anti-tumour effect. In further studies, synthetic oligonucleotides derived from BCG gene sequences were shown to be capable of inducing immunostimulatory effects (both in vitro and in vivo). The authors of these studies concluded that certain palindromic sequences, including a central CG motif, carried this activity. The central role of the CG motif in immunostimulation was later elucidated in a publication by Krieg, Nature 374, p546 1995. Detailed analysis has shown that the CG motif has to be in a certain sequence context, and that such sequences are common in bacterial DNA but are rare in vertebrate DNA. The immunostimulatory sequence is often: Purine, Purine, C, G, pyrimidine, pyrimidine; wherein the CG motif is not methylated, but other unmethylated CpG sequences are known to be immunostimulatory and may be used in the present invention.

In certain combinations of the six nucleotides a palindromic sequence is present. Several of these motifs, either as repeats of one motif or a combination of different motifs, can be present in the same oligonucleotide. The presence of one or more of these immunostimulatory sequences containing oligonucleotides can activate various immune subsets, including natural killer cells (which produce interferon γ and have cytolytic activity) and macrophages (Wooldrige et al Vol 89 (no. 8), 1977). Other unmethylated CpG containing sequences not having this consensus sequence have also now been shown to be immunomodulatory.

CpG when formulated into vaccines, is generally administered in free solution together with free antigen (WO 96/02555; McCluskie and Davis, *supra*) or covalently conjugated to an antigen (WO 98/16247), or formulated with a carrier such as aluminium hydroxide ((Hepatitis surface antigen) Davis *et al. supra* ; Brazolot-Millan *et al., Proc.Natl.Acad.Sci.*, USA, 1998, 95(26), 15553-8).

Such immunostimulants as described above may be formulated together with carriers, such as for example liposomes, oil in water emulsions, and or metallic salts, including aluminium salts (such as aluminium hydroxide). For example, 3D-MPL may be formulated with aluminium hydroxide (EP 0 689 454) or oil in water emulsions (WO 95/17210); QS21 may be advantageously formulated with cholesterol containing liposomes (WO 96/33739), oil in water emulsions (WO 95/17210) or alum (WO

98/15287); CpG may be formulated with alum (Davis *et al. supra* ; Brazolot-Millan *supra*) or with other cationic carriers.

Combinations of immunostimulants are also preferred, in particular a combination of a monophosphoryl lipid A and a saponin derivative (WO 94/00153; WO 95/17210; WO 96/33739; WO 98/56414; WO 99/12565; WO 99/11241), more particularly the combination of QS21 and 3D-MPL as disclosed in WO 94/00153. Alternatively, a combination of CpG plus a saponin such as QS21 also forms a potent adjuvant for use in the present invention.

Thus, suitable adjuvant systems include, for example, a combination of monophosphoryl lipid A, preferably 3D-MPL, together with an aluminium salt. An enhanced system involves the combination of a monophosphoryl lipid A and a saponin derivative particularly the combination of QS21 and 3D-MPL as disclosed in WO 94/00153, or a less reactogenic composition where the QS21 is quenched in cholesterol containing liposomes (DQ) as disclosed in WO 96/33739.

A particularly potent adjuvant formulation involving QS21, 3D-MPL & tocopherol in an oil in water emulsion is described in WO 95/17210 and is another preferred formulation for use in the invention.

Another preferred formulation comprises an aluminium salt together with a CpG oligonucleotide.

In a further aspect of the present invention there is provided a method of manufacture of a vaccine formulation as herein described, wherein the method comprises mixing a protein according to the invention with a suitable adjuvant and, optionally, a carrier.

Particularly preferred adjuvant and/or carrier combinations for use in the formulations according to the invention are as follows:

i) 3D-MPL + QS21 in DQ

ii) Alum + 3D-MPL

iii) Alum + QS21 in DQ + 3D-MPL

iv) Alum + CpG

v) 3D-MPL + QS21 in DQ + oil in water emulsion

The invention is illustrated in the accompanying examples and Figures:

EXAMPLES:

Example 1: Cloning and expression of recombinant-SAG3

Bacterial strains

The DH5αF'IQ *Escherichia coli* strains (Bethesda Research) was used for bacterial transformation and recombinant plasmid propagation as described by Maniatis et al. (1982). For protein production, recombinant plasmid was transferred to BL21(DE3) and AD494(DE3) *E. coli* strains (Novagen).

Oligonucleotides synthesis

Oligonucleotides were synthesized by the solid-phase phosphoramidite method (Beaucage and Caruthers, 1981) on an Applied Biosystems Synthesizer model 394).

Polymerase chain reaction

Polymerase chain reaction (PCR) amplification was performed on a *T. gondii* tachyzoites RH strain λgt11 cDNA library (Saavedra et al., 1991). The choice of the primers was based on the published sequence (Cesbron-Delauw et al, 1994). Oligonucleotides containing respectively, a *Hind*III and a *Eco*RI restriction sites 5'GGATCAAGCTTCCATGCAGCTGTGGCGGCGCAGAGC3' and 5'TGATCGAATTCTTAGGCAGCCACATGCACAAGGAGA3' were used to amplify the sequence encoding amino acids 1-385 of SAG3, DNA was amplified in a 50 µl reaction mixture containing 10mM Tris-HCl (pH 8,3), 2mM KCl, 0,01% wt/vol gelatin, 200µM of each deoxynucleoside triphosphate, 20 pmol of each primer, 1 U of Taq polymerase and cDNA. Samples were amplified for 30 cycles in a DNA thermal cycler (Perkin-Elmer Cetus). After an initial 10 min denaturation at 94°C, each cycle consisted of 1 min at 95°C, 2 min at 55°C and 3 min at 72°C. At the end of the 30 cycles of amplification, a primer extension was continued for 10 min at 72°C. The PCR products were analysed after gel electrophoresis on a agarose gel. The amplification of the sequence encoding amino acids 1-359 of anchor-less SAG3 was realized in a similar way but with the C-terminal oligonucleotide 5'TGATCGAATTCTTATTCTGTTCCAGCTTGACTTTCC3'.

Plasmid construction

The amplified DNA fragment was digested by *Hind*III and *Eco*RI endonucleases before its insertion in the pUC19 (New England Biolabs) previously opened with the same enzymes. The sequence of the amplified DNA was confirmed by dideoxy sequencing.

Additional unique cloning sites (*Sna*BI, *Stu*I and *Sma*I) were introduced in the pET-b (Novagen) vector previously opened by *Nde*I and *Bam*HI and ligated with *Nde*I-*Bam*HI synthetic linker obtained by annealing the oligonucleotides 5'TATGTACGTAGGCCTCGAGGCCCGG3' and 5'GATCCCGGGCCTCGAGGCCTACGTACA3'. The resulting plasmid pET-15b-linker, was then cut by SmaI and desphosphorylated to introduce a bp *Bsa*WI-*Eco*RI blunt-ended with T4 DNA polymerase. The resulting plasmid, pNIV4716, contains the sequence encoding the amino acids 40-359 of SAG3 downstream.

Culture conditions

PNIV4716 was introduced into the *E. coli* strain BL21 (DE3) or AD494 (DE3) (Novagen). All cell cultures were performed as described in the manufacturer's instruction leaflet (Novagen). Briefly, single colonies were grown overnight at 37°C in 869 medium in presence of 100 µg/ml ampicilin and 30 µg/ml kanamycin (only for the AD494 (DE3) derived strains). Cells were then diluted 100- to 200-fold and allow to grow at 37°C to an optical density between 0,6 to 0,8. Isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 100 mM. Following another 2 h 30 of growth, cells were harvested by centrifugation (20 min at 2060g, 4°C).

Immunological tools

Peptide synthesis and purification

Two peptides derived from SAG3 were chosen according to predictive algorithms for B-cell epitopes (Chow and Fasman, 1978, Hopp and Woods, 1981; Kyte and Doolittle, 1982): Peptides IDAKDKGDCERNNKGFLT (aa 122-138) and SGDSVDPQKCSPQSLT (aa 254-269). They were synthesized by the merrifield solid-phase method on a fully automated peptide synthesizer (ABI model 430 A,

Foster City, CA), according to the tert-butyloxycarbonyl/trifluoracetic acid (τboc/TFA) strategy (Kent, 1988). After synthesis, peptides were deprotected and cleaved from the resin by hydrogen fluoride. The crude peptides were purified by gel filtration on TSK HW 40s (Merck, Rahway, NJ) and reverse-phase high pressure liquid chromatography (RP-HPLC + on Nucleosil C_{18}. Peptides were then checked for homogeneity by RP-HPLC on Vydac C_{18} and thin-layer chromatography, and for identity by amino-acid analysis after total acid hydrolysis. Peptides were conjugated to the tetanus toxoïd with coupling agent as carbodiimide .

Immunisation with peptides

Rabbits were subcutaneously immunized at 1 month intervals using 500 µg of conjugated peptide emulsified in complete Freund's adjuvant for the first injection and Freund's incomplete adjuvant for the second. Rabbits were bled 3 weeks after the second injection.

Results

The sequence coding for SAG3 (385 amino acid residues) was recovered by PCR amplification from a λgt11 tachyzoite cDNA library. This sequence (Figure 1a), verified by automatic dideoxysequencing, shows some differences with one previously found by Cesbron et al (6) (Figure1b). Indeed, 17 bases were different which do not result from sequencing error or mistake issues from PCR amplification. Sequencing was realized on several different fragments obtained from PCR amplification. Each time, these mutations were found. The deduced amino acid (Figure 2a) shows 97.4% homology with one found by Cesbron et al (6). Indeed, 10 amino acid residues were different (Figure 2b). SAG3 carries a C-terminal region coding for a stretch of hydrophobic amino acids (residues 360-385) which will be removed upon addition of a phosphatidylinositol-containg glycolipid anchor. As these hydrophobic segments are not present in the mature SAG3, they were removed prior to fusion with the N-terminal His-tag in derived pET-15b vector. The resulting plasmid, pNIV4716, carries under the control of the strong bacteriophage T7 promoter, the fused sequences of the His-Tag® followed by a thrombin site and anchor-less SAG3 (amino acids 40-359).

Transformants were tested for expression; cells were lysed by two passages through French Press. The lysate was centrifuged at 15000g for 10 min. The pellet was then treated with 20nM Tris-HCl pH 7,5 containing 8 M urea overnight at 4°C. After ultracentrifugation, the both supernatants were analysed in reducing conditions, on SDS-PAGE and proteins were detected by immunoblotting using antipeptide antibodies targeting SAG3 residues 122-138 or 254-269, respectively. A protein having apparent molecular mass of 46 kDa was only detected in the urea supernatant. As previously observed for SAG1, the non-reduced recombinant SAG3 protein migrated with a higher mobility than its reduced equivalent, (Figure 3), Indeed, the unreduced recombinant SAG3 was immunodetected as an apparent molecular mass of 38,5 kDa with *T.gondii* specific human IgG antibodies or with antibodies against residues 122-138 of SAG3 (Figure 3). Thus, the presence of additional 28 amino acids at the N-terminal end (MGSSHHHHHHSSGLVPRGSHMYVGLEAP) does not seem to have significant effect on the conformation of the molecule as shown, recognition by *T. gondii* specific human antibodies.

A higher expression of SAG3 was observed with the thioredoxin reductase deficient strain AD494(DE3) allowing the formation of disulfide bonds in the *E. coli* cytoplasm.

Example 2: Purification of recombinant SAG-3

E. coli, containing the recombinant expression vector pNIV4716 from an overnight culture diluted 1:100, were grown at 37°C in 869 medium supplemented with 100 µg/ml ampicillin to an optical density between 0.4 and 0.6 at 600 nm. The $(His)_6$-SAG-3 expression was induced by addition of Isopropyl β-D-thiogalactoside (IPTG) to a final concentration of 0.3 mM for 4 h at 37°C. Bacteria were harvested by centrifugation, resuspended in 20mM Tris-HCl pH 7.5, containing 1mM aprotinin and AEBSF, and broken by two passages through a Cell Disrupter at a pressure of 18 Kbars. The lysate was centrifugated at 1400g for 30 min. The inclusion bodies was washed two times with 20mM Tris-HCl pH 7.5 and $(His)_6$-SAG-3 was solubilized with PBS pH 7.5 containing 500mM NaCl and 6M urea for 2h at 4°C. The suspension was centrifugated at 30000g for 30 min. The supernatant was applied onto a Ni^{2+}-NTA column (Qiagen) equilibrated in PBS pH 7.5 containing 500mM NaCl and 6M urea. After washing the column with the equilibrating buffer, $(His)_6$-SAG-3 was directly renaturated onto the column by passage of PBS containing 500mM NaCl through the resin. $(His)_6$-SAG-3 was eluted by addition of 100-200mM Imidazole in the renaturing buffer.

Results

The purity of $(His)_6$-SAG-3 was checked by SDS-PAGE. Protein was visualized by Coomassie blue staining: the protein migrated as a band of 43kD and its purity was greater than 90%.

Example 3: Interaction of recombinant SAG3 with cellular proteoglycans

Cell line

Chinese Hamster Ovary cells (CHO-K1, ATCC) were grown in Glasgow Minimum Essential Medium (GMEM, Gibco) supplemented with 5 % fetal calf serum (FCS, Gibco).

Parasites

The virulent C56 strain of *T.gondii* was maintained in our laboratory by intraperitoneal passages in BalbC mice. Briefly, each mouse was injected with 10^4 parasites in 400µl PBS pH 7.3. Six days later, mice were sacrified and between 4 and 8 10^6 tachyzoites were harvested after a peritoneal lavage with 10ml PBS. Tachyzoites were filtered on 3µm polycarbonate membranes and washed twice with PBS. Cellular debris were removed by centrifugation for 20 min at 1000 g. The concentration of parasite was determined by enumeration in a Thoma counting chamber at 400x magnification.

Reagents

Bovine serum albumin (BSA), heparin, chondroitin sulfate A (CS A), dermatan sulfate (DS) were from Sigma. Dextran sulfate, Heparin Sepharose CL-4B and Sephadex G-25 were from Amersham Pharmacia Biotech.
Soluble recombinant SAG-3 was purified from *Escherichia coli* culture as previously described.

Protein iodination

300 µg purified recgSAG-3 were incubated with 1 mCi $Na^{125}I$ (Amersham) and one Iodobead (Pierce) for 8 min. Free ^{125}I was removed by gel filtration on a 5 ml Sephadex G-25 column equilibrated in PBS. The trichloroacetic acid-precipitable radioactivity was at least 90 % of the total.

Radiolabelling of tachyzoites

About 10^7 tachyzoites in 300 µl of PBS were radiolabelled with 1 mCi $Na^{125}I$ and one Iodobead for 8 min. Free ^{125}I was removed by centrifugation. The specific activity of labelled parasites was 12000 c.p.m./p.f.u.

Binding of recombinant SAG-3 and recombinant SAG1 to immobilized heparin

100 µg of recSAG-1 or recSAG-3 were loaded onto 2 ml heparin Sepharose columns equilibrated with PBS in presence or absence of 1 mg/ml heparin . The columns were washed with PBS and eluted with 2 mg/ml heparin in PBS. Fractions were analyzed by SDS PAGE. Proteins were detected by Coomassie blue staining.

Binding of recombinant SAG-3 to cell surfaces

Confluent monolayers of CHO-K1 (containing approximatively $5x10^5$ cells) growing in 24-well dishes were rinsed with PBS and incubated with 500 µl of buffer A (PBS 98 %, FCS 2 %, BSA 0.5 %) for 1h at 4°C. The medium was removed and the cells were incubated with 2µg/well of ^{125}I-recSAG-3 in 300 µl of buffer A for 2h at 4°C. After incubation, cells were washed four times with buffer A followed by lysis in 500 µl of 1 % SDS and 1 % Triton X-100 in PBS. The cell-associated radioactivity was counted in a Wallac 1470 Wizard auto γcounter. Each experiment was performed in quintuplicate.

Non specific binding of ^{125}I-recSAG-3 was determined in the presence of a 60-fold excess of unlabeled proteins.

For competition assays, ^{125}I-recSAG-3 was mixed with various concentrations of heparin, dextran sulfate, chondroitin sulfate A or dermatan sulfate and incubated with the cells as described above. The non specific background binding was substracted.

The residual SAG-3 binding was expressed as the ratio of cell-associated radioactivity in the presence of glycoaminoglycans to the value obtained in the absence of competitors.

Inhibition of sulfation by mammalian cells

CHO-K1 cells (containing approximatively $5x10^5$ cells) were grown in 24-well dishes in G-MEM medium supplemented with 5% FBS. $NaClO_3$ or NaCl as a control was added to a final concentration of 30mM and cells were grown for an additional 20 or

48h. Cells were washed twice with PBS and assayed for recSAG-3 binding as described above.

Parasite attachment assay

Confluent monolayers of CHO-K1 cells (containing approximatively $5x10^5$ cells) grown in 24-well dishes were treated as previously described for the recSAG-3 binding assays. Cells were incubated with 10^5 radiolabelled parasites in 300 µl of buffer A for different period of time at 4°C.

In another set of experiments, cells were preincubated with various concentrations of recSAG-3 for 90 min at 4°C and the radiolabelled tachyzoites were then added to cells for 2h.

In all cases, the parasite inoculum was removed and the cells were washed four times with buffer A followed by lysis in 500 µl of 1 % SDS and 1 % Triton X-100 in PBS. The percentage of tachyzoites bound to cells was determined by counting the residual cell-associated radioactivity. Results are the mean of quintiplicates.

Results

Recombinant SAG3 binding to immobilized heparin

The initial interaction between *Toxoplasma gondii* and susceptible cells involves adsorption to cell surface proteoglycans, particularly heparan sulfate proteoglycans. This interaction was mimicked by using heparin sepharose column to assess the binding of SAG-1 or SAG-3 to heparin. Recombinant SAG-1 (recSAG-1) and SAG-3 (recSAG-3) were chromatographed onto heparin Sepharose under physiological conditions (PBS, pH 7.3). The column was washed with three volumes PBS and bound proteins were eluted with soluble heparin. Under these conditions, recSAG-3 bound to heparin Sepharose and could be eluted from the resin with soluble heparin (Fig.3.A). In contrast, no recSAG-1 could interact with heparin (Fig.3.B). The heparin binding of recSAG-3 was specific as addition of 100µg/ml heparin could inhibit nearly all the binding of rec-SAG-3 to heparin Sepharose (Fig 3.C). Altogether, these results suggest that SAG-3 could specifically interact with cellular proteoglycans. The interaction of recSAG-3 with the cell surface was studied subsequently.

RecSAG-3 binding to cell surface proteoglycans.

The interaction of recSAG-3 with CHO K1 cells was measured to determine the glycoaminoglycan specificity of its binding to the cell surface. All the binding experiments were performed at 4°C to minimize the effects of receptor internalization and recycling. Radioiodinated recSAG-3 (2µg/well) was incubated with cells in presence or absence of a 60-fold excess of unlabeled recSAG-3. As shown in Figure 4, ^{125}I-recSAG-3 binds to CHO K1 cells in a specific manner. Indeed, binding of radiolabeled recSAG-3 to CHO cells was inhibited up to 80% by unlabeled recSAG-3. This inhibition was also observed when binding assays were performed with cells preincubated with an excess of unlabeled recSAG-3.

It must be pointed out that recSAG-3 reached maximum binding whithin 2h of incubation at 4°C (data not shown).

These results provided evidence that recSAG-3 binds to cells through interactions with specific ligand(s).

To determine whether SAG-3 is able to bind to cell surface proteoglycans, exogenous soluble glycoaminoglycans were examined for their ability to inhibit the ^{125}I-recSAG-3 binding to CHO-K1 cells. Competitions were performed with soluble heparin, chondroitin sulfate A, dermatan sulfate or dextran sulfate (0 to 50 µg/ml). As shown in Figure 5 heparin was the most effective inhibitor of ^{125}I-recSAG-3 attachment. Heparin at 2µg/ml inihibited the SAG-3 binding by up to 90%. Dermatan sulfate but not chondroitin sulfate A inihibited the recSAG-3 adsorption to CHO cells in a dose-dependent manner. On the contrary, addition of dextran sulfate at low concentration (0 to 5µg/ml) increased the SAG-3 binding by about two-fold, the effect being dose-dependent with maximal stimulation at 5µg/ml. The SAG-3 binding was found to be only competed by dextran sulfate at concentration higher than 10µg/ml.

These results indicate that SAG-3 interacts specifically with cell surface proteoglycans.

Proteoglycan sulfation is required for maximal recSAG-3 binding to cells

As glycoaminoglycans are sulfated polysaccharides, the sulfate groups present on the cell surface could be essential for the recSAG-3 binding. To test this, binding experiments were performed with CHO-K1 cells pretreated with sodium chlorate, a competitive inhibitor of ATP-sulfurylase which consequently reduce sulfation of proteins and proteoglycans. Incubation of cells with sodium chlorate reduced the

recSAG-3 binding by more than 75% as compared with control cell (Figure 6). The effect of chlorate was not a result of CHO-K1 growth in an higher ionic strength medium, as pretreatment of the cells with sodium chloride at similar concentration had no effect on recSAG-3.

RecSAG-3 inhibits *Toxoplasma gondii* attachment to cells

To assess the relevance of the interactions between SAG-3 and cell surface proteoglycans at the level of *T.gondii* infection, we next investigated whether recSAG-3 could inhibit parasite adsorption. Prior to this study, a number of preliminary experiments were performed to optimize the conditions for the parasite binding assay. We found that the experimental conditions previously optimized for SAG-3 binding could be applicable to radioiodinated tachyzoites (data not shown). The attachment of radiolabeled parasites is illustrated in Figure 7. The cell-associated radioactivity increased in a time-dependent manner, the parasite reaching maximum binding within 2h of incubation at 4°C. Tachyzoites demonstrated a markedly lower relative attachment rate when cells were preincubated with xμg/ml recSAG-3 for 90 min. RecSAG-3 inhibited the parasite adsorption by up to 40%. In another set of experiments, CHO-K1 cells were pretreated with various concentrations of recSAG-3 (from 2 to 200μg/ml) for 90min at 4°C and radiolabeled tachyzoites were added for further 90 min at 4°C. RecSAG-3 blocked the parasite adsorption by up to 75%, to comparable extents at all doses tested. These results provided direct evidence that the interaction of recSAG-3 with the cell blocks a step in *T.gondii* attachment and that recSAG-3 binds to the same receptor as *T.gondii* SAG-3. Taken together, our results show that SAG-3 mediates in the *T.gondii* adsorption to cell surface proteoglycans.

Example 4: Immunogenicity of recombinant SAG3

a) Guinea pigs

T.gondii

The C56 medium-virulent strain of toxoplasma gondii (kindly supplied by ML Darde, CHU Limoges), maintained by passage of infective brain homogenate in the peritoneum of BalbC mice, was used for experimental infections in Durkin-Hartley guinea pigs.

Vaccination procedure

Groups of 20 guinea pigs were immunized three times at 3 weeks intervals using either 5 µg of recombinant SAG3 formulated with adjuvant 1 or adjuvant 1 alone (SC route). SAG3 was produced in *E.coli* and purified according to the procedure described in the present patent.

Adjuvant 1 comprises 3D-MPL and a non-reactogenic form of QS21 as described in WO96/33739.

Measurement of antibody response

Two weeks after the last immunization, animals were bled and sera were tested for the presence of anti-SAG3 IgG antibodies in an enzyme-linked immunosorbent assay (ELISA) using recombinant SAG3 as coating reagent (100 ng/well). Serial dilutions of each serum were tested and titer was expressed as the reciprocal of the dilution giving 50% of the maximal optical density.

Congenital infection model

Before antigen injection, all guinea pigs were monitored for absence of seroreactivity against Toxoplasma. Females were mated with males for breeding three weeks after the last immunization; challenge infection using 5.10^5 tachyzoïtes (ID route) followed 7 weeks later.

Infectious status of pups delivered from guinea pigs was evaluated in a mouse assay, pups were sacrified within 48 hours following delivery, each brain was homogenized

in 1 ml of PBS and intra peritoneally injected into two female BalbC mice (0.5 ml each). Mice that did not survive from 21 days onwards after brain homogenate injection were considered infected and their mortality indicated the infection status of the pups ; we assessed that a pup was infected once one of the two injected mice died.

b) Mice

Vaccination procedure

Four groups of twelve mice were immunized (SC route) three times at three weeks intervals with 5 µg of rec-SAG3 formulated either with adjuvant 1 (group 1) or adjuvant 2 (group 2). Group 3 and group 4 received respectively adjuvant 1 (group 3) or adjuvant 2 (group 4). First (S1) and second (S2) bleeding were respectively performed 3 weeks after the second immunization and) 3 weeks after the last one.

Adjuvant 2 comprises 3D-MPL and QS21 in an oil in water emulsion comprising squalene, α-tocopherol and Tween-80, prepared as described in WO95/17210.

Measurement of antibody response

IgG, IgG1 and IgG2a titers in the sera were determined by an ELISA using plates coated with 100 ng per well of rec-SAG3. Total IgG were detected using a phosphatase conjugated goat anti-mouse. IgG1 and IgG2a were detected with a biotine-conjugated rat anti mouse IgG1 and IgG2a respectively followed by streptavidin alkaline phosphatase conjugate. Serial dilutions of each serum were tested and titer was expressed as the reciprocal of the dilution giving a signal 50% of the maximal optical density.

Splenocyte proliferation assay

Spleens were disrupted to obtain a single-cell suspension, followed by lysis of erythrocytes in 0.83% NH_4Cl-0.01 M Tris-HCl. The remaining splenocytes were washed and plated in 96-well plates at $4x10^5$ cells per well in RPMI 1640 medium supplemented with 1% mouse serum, 25 mM HEPES (N-2-hydroxyethylpiperazine-N'-2 ethanesulfonic acid), 2mM L-glutamine, 1 mM sodium pyruvate, 5.10^{-5}mM β-mercaptoethanol and 50 IU/ml penicilline-streptomycine. Cells were incubated in the

presence of different concentrations of recSAG3 (10, 1, 0.1 µg/ml). After 4 days at 37°C, cells were pulsed with 1 µCi per well of (^{3}H)-thymidine. 16 hours later, they were harvested and incorporated radioactivity was determined using a scintillation counter (Wallac, 1450 Micro Beta).

c) **Humans**

Measurement of antibodies against recombinant SAG3

Heparinized blood from donors was centrifuged. The presence of specific anti-SAG3 antibodies was detected using an enzyme-linked immunosorbent assay (ELISA). 96 Microwells plates were coated overnight at 4°C with 100 ng per well of purified rec-SAG3. Wells were washed five times with Tris-buffered saline (TBS)-0.1% Tween 80 and saturated in the same buffer supplemented with 1% bovine serum albumine (BSA) for 1 hour at 37°C. Plates were then incubated with serial dilutions of sera (1 hour at 37°C). Plates were washed as described above and alkaline phosphatase-labelled goat anti-human IgG antibody diluted 1/7500 was used as the secondary antibody (1h at 37°C). After washing, immune complexes were developed with p-nitrophenyl phosphate as chromogenic substrate. Absorbance at 415 nm was measured in a microplate reader (Biorad). Titer was expressed as the reciprocal of the dilution giving one-half the maximal optical density.

Results

a) Guinea pigs

IgG response of guinea pigs immunized with rec-SAG3

Antibodies responses were determined by enzyme-linked immunosorbent assay (ELISA). The geometrical mean was 38578 with values between 8764 and 126400. The titers in the mock-immunized group were below the detectable level.

Congenital transmission

After challenge, 15 SAG3- and 16 mock-immunized guinea pigs produced respectively 52 and 58 pups ; of those 4 and 20 respectively were excluded for further analyse because, in a precedent experiment, we observed that stillborn pups or pups retrieved from dead mother were always negative in the mouse assay even if they

originated from the mock-immunized group, probably due to parasite inactivation. After exclusion, 48 and 38 pups, originated from 15 and 11 litters respectivelly, were analysed.

Protection against vertical transmission was observed and summarized in Table 1

Table 1

Read out	SAG3 immunized	mock-immunized
Number of litters	15	11
Overall mortality in mice injected with brain's pup	53%	60%
Number of litters without infected pups	3	1
Number of litters partially infected	10	6
Number of litters fully infected	2	4
Number of pups	48	38
Proportion of infected pups	56	68

b) Mice

Antibodies

The mean titers were presented on Figure 8 (IgG) and Figure 9 (IgG1 and IgG2a). In both groups (1 and 2), a specific IgG response was generated after two immunizations, IgG1/IgG2a titers were the same for the two formulations (1/5), but adjuvant 2 induced a stronger humoral response than adjuvant 1.

Lymphoproliferation assay

Three weeks after the second immunization, mice were sacrified and the splenocytes were isolated to assess the systemic proliferative response to rec-SAG3. T cell proliferation was expressed as the stimulation index (SI), calculated as the ratio of mean cpm in SAG1 stimulated cells to control cells. A positive response was defined as a SI >2 (Figure 10). We observed proliferation for the 3 concentrations of antigen tested and whatever the adjuvant used. The weakest response observed with the highest concentration of rec-SAG3 was probably due to the presence of imidazole in the SAG3 preparation.

c) **Human**

Antibodies

Toxoplasma serology was performed by Bigaignon,UCL (ELISA Bio-Merieux). Sera with titers lower than 10 IU/ml were considered as negative (table 2, second column). *T.gondii*-seropositive or –seronegative human sera were tested for IgG directed to rec-SAG3 (ELISA test). A good correlation was observed between the values of,the clinical test and those obtained in the ELISA test against SAG3.

Serum	IgG against *T.gondii*	IgG against rec-SAG3
1	300	3180
2	196	930
3	186	860
4	85	440
5	81	170
6	44	1500
7	32	110
8	30	280
9	24	175
10	21	0
11	16	70
12	10	0
13	4	0
14	0	0
15	0	0

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9. Mineo,J.R.and Kasper, L.H. (1994). Attachment of Toxoplasma gondii to host cells involves major surface protein SAG1 (p30). Exp.Parasitol.79: 11-20.

10. Grimwood, J. and Smith J.E. (1992). Toxoplasma gondii: the role of a 30-kDa surface protein in host cell invasion. Exp.Parasitol. 74:106-111

11. Tomavo,S.(1996). The major surface proteins of Toxoplasma gondii: Structures and functions. 45-54. In Toxoplasma gondii. Heidelberg: Springer. U.Gross (Editor).

SEQ ID No:1

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 289 ATGCAGCTGTGGCGGCGCAGAGCAGCAGGTCCCGCGAGCCTGGGGAGGCA 338
 339 GTCTTTGCCGCTCGGGTGTTTTTTCGCGGCTTTTGGTTTGTGCGTGTTGT 388
 389 CTGCGATCTTGGGAACCGGAGAGCACGGACTGTTCGTCGCCGCAGGTAAA 438
 439 TCGAGAAGTAAGATAACTTATTTTGGCACGCTCACTCAGAAGGCTCCGAA 488
 489 CTGGTACCGCTGCTCTTCAACGAGGGCGAAAGAAGAGGTCGTAGGACATG 538
 539 TGACGCTGAACAAAGAGCACCCTGATATGACAATTGAATGCGTCGACGAC 588
 589 GGCTTGGGCGGAGAGTTTTTGCCGCTCGAAGGCGCGACGTCGTCGTACCC 638
 639 GCGAGTATGTCACATTGATGCCAAGGACAAGGGCGACTGCGAGCGCAACA 688
 689 AGGGCTTTCTGACCGACTACATACCGGGCGCGAAGCAGTACTGGTACAAG 738
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 789 AGTTCCTTGGATATTCCTTCCGCCCGCCAAGCAGCGATACAAGGTTGGAT 838
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 889 ACGCCGCCAATGGTCGAAGGCAAGAGAGTGACCTGCGGGTACCCCGAGTC 938
 939 CGGCCCCGTGAATCTCGAGGTGGACTTGTCAAAGGACGCGAACTTTATCG 988
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1089 CCTGACGAACATTTTTTATGACTACAGCTCTTCGTGGTGGAAGGGGAAAC 1138
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1239 GCCCTTCTGCAACGTCAAAGTGAGAGTTGCCGGGAACCCCAGAAAGTGGG 1288
1289 GGAGAGGCGGAGGCGGCCATCCAGGAAGCGGAGGATCGCAGCCGGAAACT 1338
1339 GACGGGGAAACTCAAGCTGGAACAGAAAGTTCAGCCGGCGCGAGTTCGCG 1388
1389 AATGGCTTCCGTTGCCCTGGCGTTCCTTCTCGGTCTCCTTGTGCATGTGG 1438
1439 CTGCCTAA 1446
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SEQ ID No:2

MQLWRRRAAG PASLGRQSLP LGCFFAAFGL CVLSAILGTG EHGLFVAAGK
SRSKITYFGT LTQKAPNWYR CSSTRAKEEV VGHVTLNKEH PDMTIECVDD
GLGGEFLPLE GATSSYPRVC HIDAKDKGDC ERNKGFLTDY IPGAKQYWYK
IEKVENNGEQ SVLYKFTVPW IFLPPAKQRY KVGCRYPNHE YCFVEVTVEP
TPPMVEGKRV TCGYPESGPV NLEVDLSKDA NFIEIRCGEQ HHPQPSTYTL
QYCSGDSVDP QKCSPQSLTN IFYDYSSSWW KGKLNGPDGA TLTIPPGGFP
EEDKSFLVGC SLTVDGPPFC NVKVRVAGNP RKWGRGGGGH PGSGGSQPET
DGETQAGTES SAGASSRMAS VALAFLLGLL VHVAA*

CLAIMS

1. A vaccine composition comprising the toxoplasma protein, SAG3 or an immunogenic derivative thereof in combination with a suitable adjuvant and/or carrier.

2. A vaccine composition comprising the toxoplasma protein of SEQ ID No: 2 or an immunogenic derivative thereof in combination with a suitable adjuvant and/or carrier.

3. A vaccine composition comprising a truncated toxoplasma protein which comprises amino acid residues 40 – 359 of SEQ ID No: 2 or an immunogenic derivative thereof in combination with a suitable adjuvant and/or carrier.

4. A vaccine composition as claimed in any one of claims 1 to 3 wherein the adjuvant is a Th1-type inducing adjuvant.

5. A vaccine composition as claimed in claim 4 wherein the adjuvant comprises at least one immunostimulant chosen from the group consisting of 3D-MPL, QS21 and CpG.

6. A vaccine composition as claimed in claim 4 or claim 5, formulated with at least one carrier chosen from the group consisting of an oil in water emulsion, an aluminium salt and cholesterol-containing liposomes.

7. A vaccine composition as claimed in claim 5 or claim 6, wherein the adjuvant comprises 3D-MPL.

8. A vaccine composition as claimed in any one of claims 5 to 7, wherein the adjuvant comprises a saponin such as QS21.

9. A vaccine composition as claimed in claim 8 wherein the adjuvant is 3D-MPL, in combination with QS21 in a cholesterol-containing liposome.

10. A vaccine composition as claimed in any one of claims 7 to 9, further comprising alum.

11. A vaccine composition as claimed in claim 8 wherein the adjuvant is 3D-MPL and QS21 in an oil in water emulsion.

12. A vaccine composition as claimed in claim 5 or claim 6 wherein the adjuvant comprises CpG and alum.

13. A vaccine composition as claimed in any one of the preceding claims which further comprises another immunogenic protein or immunogenic derivative thereof.

14. A vaccine composition as claimed in claim 13 wherein the immunogenic protein is a toxoplasma protein or immunogenic derivative thereof.

15. A vaccine composition as claimed in claim 14 wherein the toxoplasma protein is the SAG 1 protein or an immunogenic derivative thereof.

16. A vaccine composition as claimed in claim 15 wherein the toxoplasma protein comprises amino acid residues 48 – 307 of SAG1 or an immunogenic derivative thereof.

17. A SAG3 protein as shown in SEQ ID No. 2 or an immunogenic derivative thereof.

18. A DNA sequence encoding a SAG3 protein, comprising the nucleotide seqeuence of SEQ ID No. 1.

Figure 1

Figure 1a: Nucleotide sequence of SAG3.

```
 289 ATGCAGCTGTGGCGGCGCAGAGCAGCAGGTCCCGCGAGCCTGGGGAGGCA 338
 339 GTCTTTGCCGCTCGGGTGTTTTTTCGCGGCTTTTGGTTTGTGCGTGTTGT 388
 389 CTGCGATCTTGGGAACCGGAGAGCACGGACTGTTCGTCGCCGCAGGTAAA 438
 439 TCGAGAAGTAAGATAACTTATTTTGGCACGCTCACTCAGAAGGCTCCGAA 488
 489 CTGGTACCGCTGCTCTTCAACGAGGGCGAAAGAAGAGGTCGTAGGACATG 538
 539 TGACGCTGAACAAAGAGCACCCTGATATGACAATTGAATGCGTCGACGAC 588
 589 GGCTTGGGCGGAGAGTTTTTGCCGCTCGAAGGCGCGACGTCGTCGTACCC 638
 639 GCGAGTATGTCACATTGATGCCAAGGACAAGGGCGACTGCGAGCGCAACA 688
 689 AGGGCTTTCTGACCGACTACATACCGGGCGCGAAGCAGTACTGGTACAAG 738
 739 ATAGAAAAGGTGGAGAACAACGGCGAGCAATCCGTTCTGTACAAATTCAC 788
 789 AGTTCCTTGGATATTCCTTCCGCCCGCCAAGCAGCGATACAAGGTTGGAT 838
 839 GCCGATACCCGAACCACGAGTATTGCTTTGTTGAGGTCACCGTCGAACCC 888
 889 ACGCCGCCAATGGTCGAAGGCAAGAGAGTGACCTGCGGGTACCCCGAGTC 938
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1039 CAGTACTGCTCAGGTGACTCGGTGGACCCGCAGAAGTGTTCGCCGCAGTC 1088
1089 CCTGACGAACATTTTTTATGACTACAGCTCTTCGTGGTGGAAGGGGAAAC 1138
1139 TGAACGGGCCTGACGGGGCAACTCTCACCATTCCACCCGGCGGGTTCCCC 1188
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1289 GGAGAGGCGGAGGCGGCCATCCAGGAAGCGGAGGATCGCAGCCGGAAACT 1338
1339 GACGGGGAAACTCAAGCTGGAACAGAAAGTTCAGCCGGCGCGAGTTCGCG 1388
1389 AATGGCTTCCGTTGCCCTGGCGTTCCTTCTCGGTCTCCTTGTGCATGTGG 1438
1439 CTGCCTAA 1446
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Figure 1b: Comparison of nucleotide sequence of SAG3 with SAG3 sequence previously published by Cesbron et al (6). Nucleotide numbering of Cesbron is conserved.

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289 ATGCAGCTGTGGCGGCGCAGAGCAGCAGGTCCCGCGAGCCTGGGGAGGCA 338
289 ATGCAGCTGTGGCGGCGCAGAGCAGCAGGTCCCGCGAGCCTGGGGAGGCA 338

339 GTCTTTGCCGCTCGGGTGTTTTTTCGCGGCTTTTGGTTTGTGCGTGTTGT 388
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389 CTGCGATCTTGGGAACCGGAGAGCACGGACTGTTCGTCGCCGCAGGTAAA 438
389 CTGCGATCTTGGGAACCGGAGAGCACGGACTGTTCGTCGCCGCAGGGAAA 438

439 TCGAGAAGTAAGATAACTTATTTTGGCACGCTCACTCAGAAGGCTCCGAA 488
439 TCGAGAAGTAAGATAACCTATTTTGGCACGCTCCTCAAGAAGGCTCCGAA 488

489 CTGGTACCGCTGCTCTTCAACGAGGGCGAAAGAAGAGGTCGTAGGACATG 538
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539 TGACGCTGAACAAAGAGCACCCTGATATGACAATTGAATGCGTCGACGAC 588
539 TGACGCTGAACAAAGAGCACCCTGATATGACAATTGAATGCGTCGACGAC 588

589 GGCTTGGGCGGAGAGTTTTTGCCGCTCGAAGGCGCGACGTCGTCGTACCC 638
589 GGCTTGGGCGGAGAGTTTTTGCCGCTCGAAGGCGGCACGTCGTCGTACCC 638

639 GCGAGTATGTCACATTGATGCCAAGGACAAGGGCGACTGCGAGCGCAACA 688
639 GCGAGTATGTCACATTGATGCCAAGGACAAGGGCGACTGCGAGCGCAACA 688

689 AGGGCTTTCTGACCGACTACATACCGGGCGCGAAGCAGTACTGGTACAAG 738
689 AGGGCTTTCTGACCGACTACATACCGGGCGCGAACAGGTACTGGTACAAG 738

739 ATAGAAAAGGTGGAGAACAACGGCGAGCAATCCGTTCTGTACAAATTCAC 788
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789 AGTTCCTTGGATATTCCTTCCGCCCGCCAAGCAGCGATACAAGGTTGGAT 838
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889 ACGCCGCCAATGGTCGAAGGCAAGAGAGTGACCTGCGGGTATCCCGAGTC 938

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1089 CCTGACGAACATTTTTTATGACTACAGCTCTTCGTGGTGGAAGGGGAAAC 1138
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1139 TGAACGGGCCTGACGGGGCAACTCTCACCATTCCACCCGGCGGGTTCCCC 1188
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1189 GAAGAAGACAAATCTTTTCTTGTCGGGTGTTCACTCACTGTGGACGGGCC 1238
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1389 AATGGCTTCCGTTGCCCTGGCGTTCCTTCTCGGTCTCCTTGTGCATGTGG 1438

1439 CTGCCTAA 1446
     ||||||||
1439 CTGCCTAA 1446
```

Figure 2

Figure 2a: Deduced amino acid sequence of SAG3

```
MQLWRRRAAG PASLGRQSLP LGCFFAAFGL CVLSAILGTG EHGLFVAAGK
SRSKITYFGT LTQKAPNWYR CSSTRAKEEV VGHVTLNKEH PDMTIECVDD
GLGGEFLPLE GATSSYPRVC HIDAKDKGDC ERNKGFLTDY IPGAKQYWYK
IEKVENNGEQ SVLYKFTVPW IFLPPAKQRY KVGCRYPNHE YCFVEVTVEP
TPPMVEGKRV TCGYPESGPV NLEVDLSKDA NFIEIRCGEQ HHPQPSTYTL
QYCSGDSVDP QKCSPQSLTN IFYDYSSSWW KGKLNGPDGA TLTIPPGGFP
EEDKSFLVGC SLTVDGPPFC NVKVRVAGNP RKWGRGGGGH PGSGGSQPET
DGETQAGTES SAGASSRMAS VALAFLLGLL VHVAA*
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Figure 2b: Comparison of deduced amino acid seqeunce with that published by Cesbron *et al.*

```
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```

Figure 3: A,B,C: SAG-3 but not SAG-1 binds to immobilized heparin

RecSAG-1 or recSAG-3 were loaded onto a heparin sepharose column. Bound proteins were eluted with soluble heparin. Chromatographic fractions were analyzed onto SDS-PAGE.

A: SAG-1: 1:molecular weigth markers, 2 : SAG-1, 3 and 4: flow-through, 5 and 6: elution with heparin. B: SAG-3 : 1: SAG-3, 2: molecular weigth markers, 3-6: flow-through, 7-10: elution with heparin. C : SAG-3 in presence of soluble heparin: 1: SAG-3, 2 : flow-through, 3: molecular weigth markers, 4: flow-through, 5-7: elution with heparin

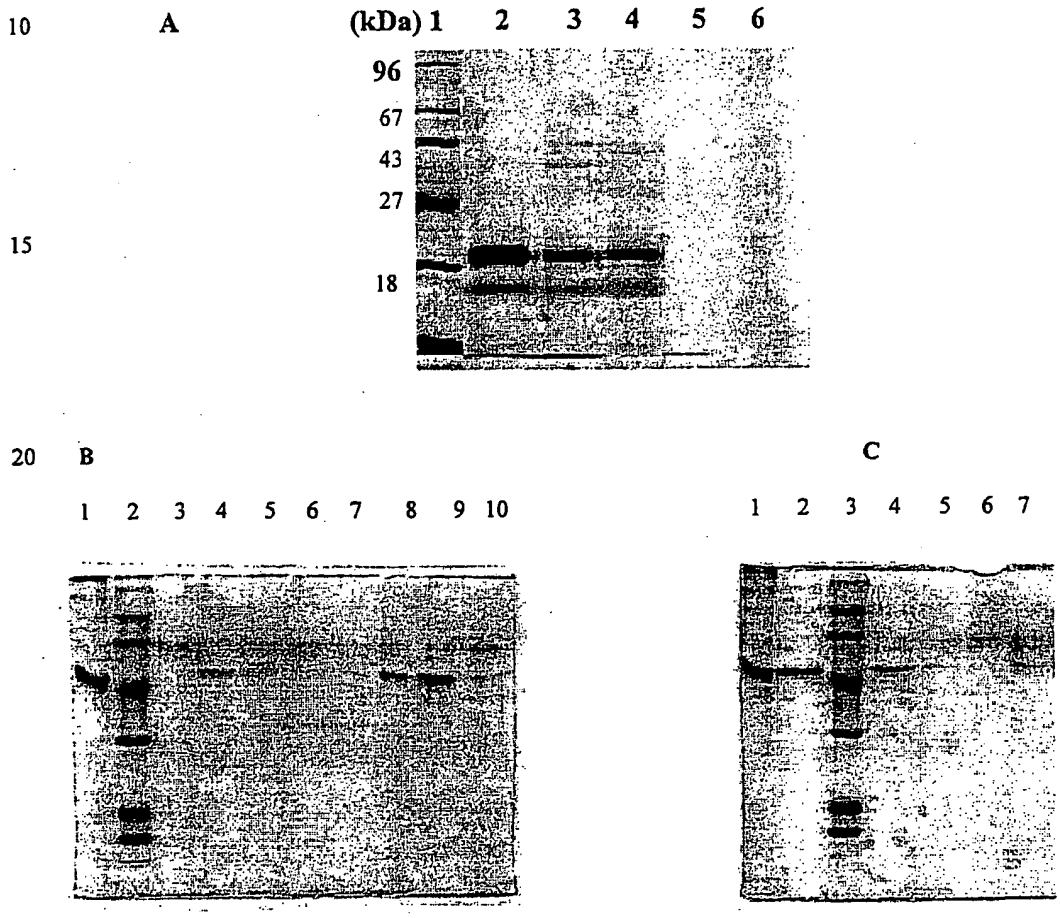
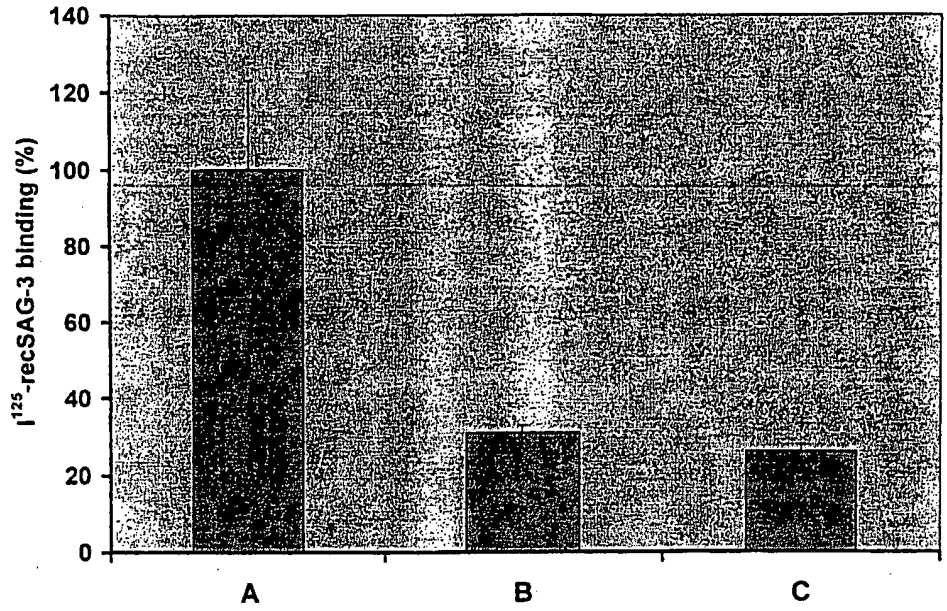


Figure 4: Binding of I^{125}-recSAG-3 on CHO cells



A confluent monolayer of CHOK-1 was incubated with 2µg/well I^{125}-recSAG-3 in the absence (A), or presence of a 60-fold excess of unlabelled SAG-3 (B). In another experiment, cells were preincubated with a 60-fold excess of unlabelled SAG-3 before addition of I^{125}-recSAG-3 (C).

Figure 5: Inhibition of recSAG-3 to CHO cells with soluble glycoaminoglycans.

CHO cells were incubated at 0 °C for 4 h with 2 µg ^{125}I-recSAG-3 in the presence of various concentrations of heparin (◆), chondroitin sulfate A (▲), dextran sulfate (○) or dermatan sulfate (❑). Cells were washed, lysed and the cell-associated radioactivity was counted. The results are the mean of quadruplicates. They are expressed as percentage of bound recSAG-3 in the absence of competitors.

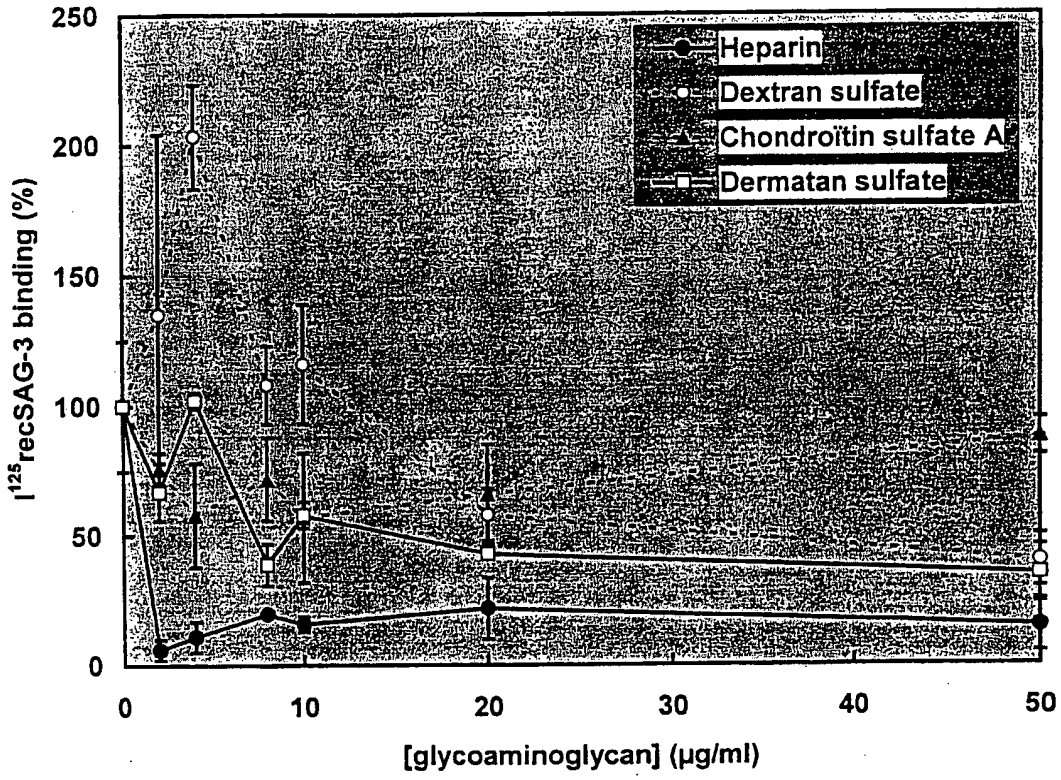


Figure 6: Inhibition of I^{125}-recSAG-3 binding by sodium chlorate

Confluent monolayers of CHOK-1 were cultivated for 20h or 48h in the presence of sodium chlorate or sodium chloride. Binding of 2µg/well of radiolabelled SAG-3 was measured.

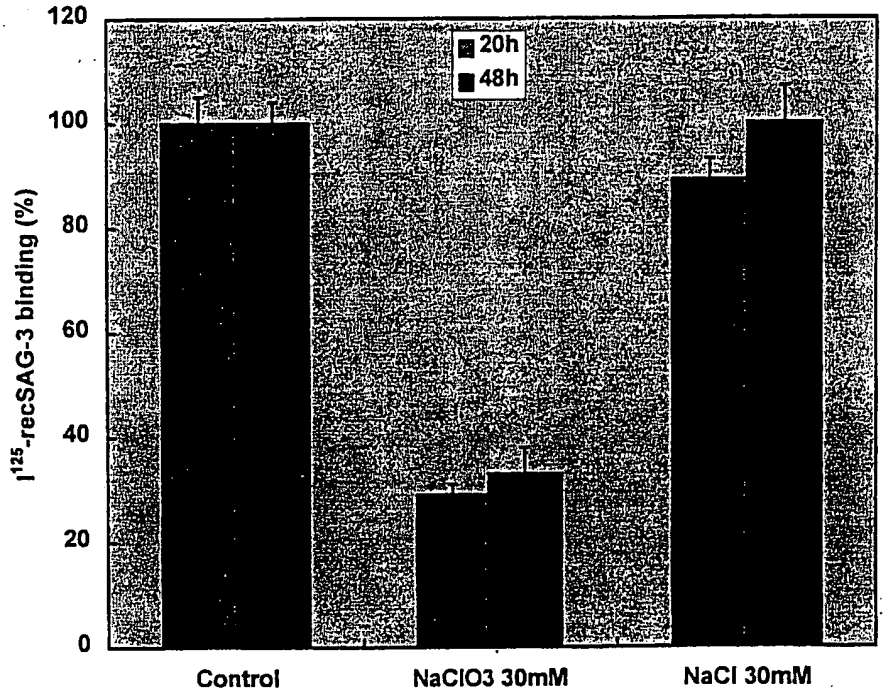
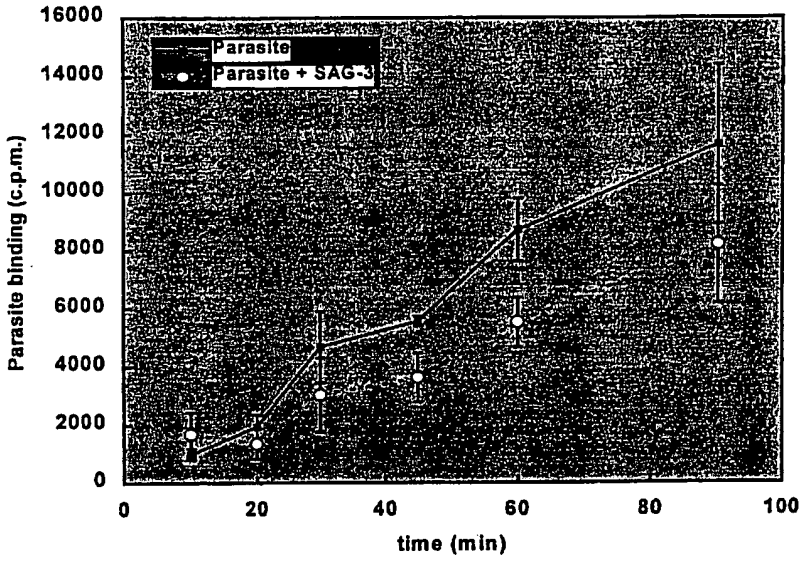


Figure 7:

Figure 7A. RecSAG-3 inhibits tachyzoites attachment to cells.

Confluent CHO-K1 were preincubated or not with 20µg/well recSAG-3 for 90min before addition of radiolabeled tachyzoites. After different times, cells were washed and cell-associated radioactivity were counted.



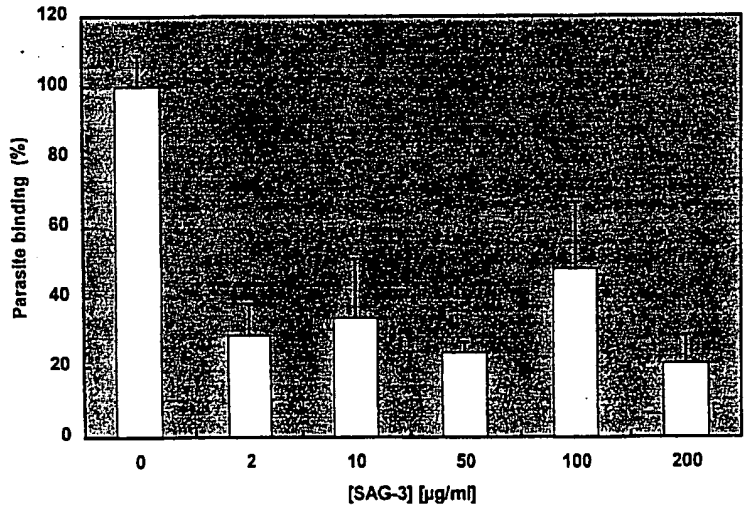


Figure 7B: CHO-K1 cells were preincubated with various concentrations of recSAG-3. Radiolabeled tachyzoites were added to cells for 90 min. Cells were washed and cell-associated radioactivity were counted.

Figure 8: IgG Titer

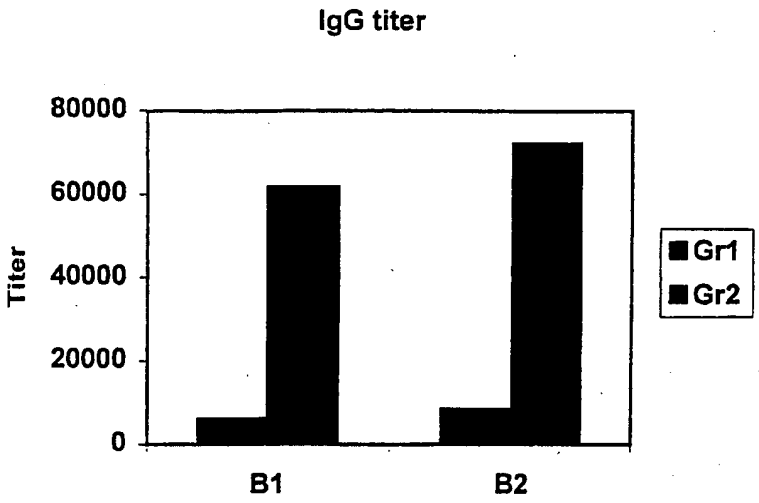
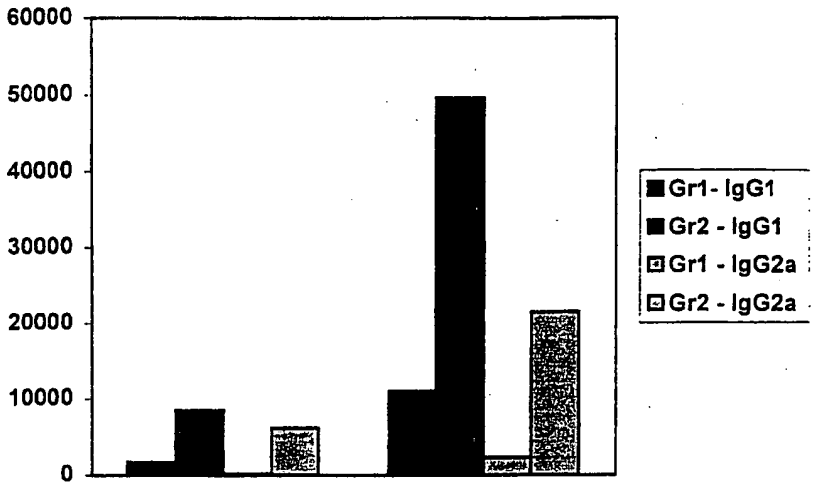


Figure 9: IgG1 and IgG2a titers



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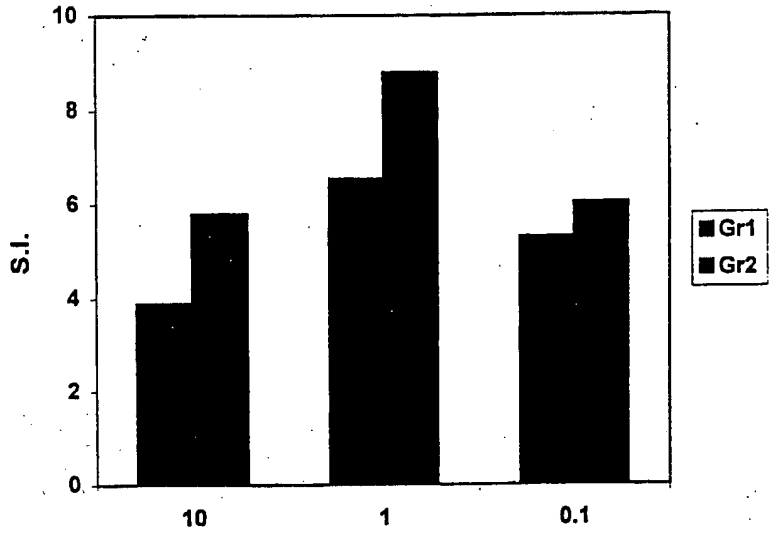

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(54) Title: VACCINE COMPOSITION COMPRISING THE TOXOPLASMA PROTEIN SAG3

(57) Abstract: The invention provides a vaccine composition comprising the toxoplasma protein, SAG3 or an immunogenic derivative thereof in combination with a suitable adjuvant and/or carrier. The invention further provides a vaccine composition which additionally comprises the toxoplasma protein, SAG1 or an immunogenic derivative thereof, or a truncated SAG1 comprising amino acid residues 48-307 of SAG1.

INTERNATIONAL SEARCH REPORT

In ational Application No
PCT/EP 00/12704

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K39/002 A61K39/39 C07K14/45 C12N15/30 A61P33/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K A61P C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
MEDLINE, CHEM ABS Data, BIOSIS, EPO-Internal, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CESBRON-DELAUW M F ET AL: "Similarities between the primary structures of two distinct major surface proteins of Toxoplasma gondii." JOURNAL OF BIOLOGICAL CHEMISTRY, (1994 JUN 10) 269 (23) 16217-22. , XP001008830 cited in the application	1-17
Y	page 16217 page 16218; figure 1	18

-/--

[X] Further documents are listed in the continuation of box C.

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Date of the actual completion of the international search	Date of mailing of the international search report
2 August 2001	24/08/2001

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

International Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MANGER I D ET AL: "The surface of Toxoplasma tachyzoites is dominated by a family of glycosylphosphatidylinositol-anchored antigens related to SAG1." INFECTION AND IMMUNITY, (1998 MAY) 66 (5) 2237-44. , XP001008344	1-17
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Information on patent family members

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PCT/EP 00/12704

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